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(54) Title: IMPAIRED BRCA2 FUNCTION IN CELLS AND NON-HUMAN TRANSGENIC ANIMALS

(57) Abstract

ScRad51, a member of the *RAD52* epistasis group in *Saccharomyces cerevisiae*, is a major component in the recombinational repair pathway employed to repair genetic damage caused by ionizing radiation. The mouse homologue of ScRad51, MmRad51, appears to have a similar function; however, the precise mechanism of action is not well understood. For ScRad51, protein:protein associations are critical for function. Therefore, the yeast two-hybrid system was used to isolate proteins that associate with MmRad51 to better understand recombinational repair in mammalian cells and mouse Brca2 was isolated. In humans, BRCA2, is a tumor suppressor gene important in the etiology of breast cancer. A phenotypic comparison between MmRad51 and Brca2-deficient embryos and cells suggest the protein:protein association is important for their function. Similar to MmRad51, Brca2 function is critical for repair of γ-radiation induced damage. In addition, a subtle mutation that removes only the small portion of Brca2 that associates with MmRad51, either directly or indirectly, exhibited a phenotype that suggests partial function. These homozygous mutant cells are viable yet hypersensitive to ionizing radiation and undergo premature replicative senescence. Cells and mice were generated with impaired Brca2 function that should prove useful as a model for tumorigenesis, a model to analyze genotoxic agents and as a tool to study premature replicative senescence.

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IMPAIRED BRCA2 FUNCTION IN CELLS AND NON-HUMAN TRANSGENIC ANIMALS

1.0. FIELD OF THE INVENTION

The present invention relates to cells and non-human transgenic animals that have been engineered to incorporate a *Brca2* gene (GenBank Accession No. U65594) that has an impaired ability to associate, either directly or indirectly, with Rad51. In particular, Brca2 activity was reduced in cells by targeted disruption of the *Brca2* gene such that the domain that codes for the Rad51, or Rad51 complex, interacting region is removed but the remainder of the coding sequence is left intact and is expressed. The engineered cells were subsequently used to generate transgenic animals that produced the altered Brca2 protein.

2.0. BACKGROUND OF THE INVENTION

Cellular DNA normally exists in a dynamic environment. Cellular functions of repair, recombination, replication and cell cycle regulation are intimately interwoven to maintain genomic stability and generate genetic diversity (reviewed by Petes *et al.*, 1991, Recombination in yeast, In: *Molecular and Cellular Biology of the Yeast Saccharomyces* (eds. J. R. Boach, J. R. Pringle, and E. W. Jones), pp. 407-521, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Drapkin *et al.*, 1994, Cell, 77:9-12; Kuhn *et al.*, 1995, Genes Dev. *9*:193-203; Friedberg, *et al.*,. 1995, DNA repair and mutagenesis, pp. 147-192, ASM Press Washington, D. C.; Li, *et al.*, 1995, Cell *83*:1079-1089). A mutation in a gene whose product is critical to any of these processes may result in a variety of clinical signs that include neurological disorders, immunodeficiency, and predisposition to cancer.

Understanding the molecular mechanisms of repair and recombination will be beneficial to understanding the etiology of disease caused by defects in these processes. The mouse is ideal for studying the dynamic nature of DNA. Similarities in human and mouse genomic constitution,

including intron-exon boundaries and the position of regulatory elements, as well as the spatial transcriptional regulation of homologous genes is remarkable (Lyon and Searle, 1989, Genetic variants and strains of the laboratory mouse, 2nd ed. Oxford University Press, Oxford). In addition, anatomical similarities between mice and humans provide the opportunity for direct physiological comparisons. Targeted disruption of genes encoding protein products such as the p53 tumor suppressor (Donehower *et al.*, 1992, Nature *356*:215-221), the mismatch repair proteins (Baker *et al.*, 1995, Cell *82*:309-319; de Wind *et al.*, 1995, Cell *82*:321-330) and the xeroderma pigmentosa complementation groups (Sands *et al.*, 1995, Nature *377*:169-173; de Vries *et al.*, 1995, Nature *377*:169-173; Nakane *et al.*, 1995, Nature *377*:165-168) have revealed striking similarities to inherited disorders in humans.

A number of different DNA repair pathways are responsible for correcting a variety of specific DNA lesions. These pathways include nucleotide excision repair, mismatch repair and double-strand break (DSB) repair. The mechanisms responsible for nucleotide excision repair and mismatch repair are fairly well understood, and mutations affecting these processes have been characterized (reviewed in Friedberg, 1992, Cell *71*:887-889; Cleaver, 1994, Cell *76*:1-4). However, the mechanisms responsible for the repair of DSBs remain poorly understood. Several inherited disorders of mammals feature defects in the repair of DSBs that are associated with hypersensitivity to ionizing radiation and immunodeficiency. These include Ataxia-Telangiectasia (AT) in humans (reviewed by Lehmann and Carr, 1995, Trends in Genet. *11*:375-377) and autosomal recessive *scid* (severe combined immunodeficiency) in mice (Roth *et al.*, 1992, Cell *70*:983-991) and in horses (Wiler, *et al.*, 1995, Proc. Natl. Acad. Sci. *92*:11485-11489).

ScRad51 is a member of the *RAD52* epistasis group in *Saccharomyces cerevisiae*, and is a major component in the yeast DSB repair pathway (by homologous recombination); a

pathway called recombinational repair (reviewed by Friedberg, *et al.*,. 1995, DNA repair and mutagenesis, pp. 523-594, ASM Press Washington, D. C.). This pathway repairs genetic damage caused by ionizing radiation. The mouse homologue of ScRad51, MmRad51, appears to have a similar function (Shinohara *et al.*, 1993, Nature Genet. 4:239-243; Lim and Hasty 1996, Mol. Cell. Biol. *16*:7133-7143); however, the precise mechanism of action is not well understood. For ScRad51, protein:protein associations are critical for function. Consequently, a yeast two-hybrid system was used to isolate proteins that associate with MmRad51 to better understand recombinational repair in mammalian cells, and mouse Brca2 was isolated (Sharan *et al.*, 1997, Nature, *386*:804-810). A phenotypic comparison between MmRad51 and Brca2-deficient embryos and cells suggest that a protein:protein association is important for their function. Similar to MmRad51, Brca2 function is critical for early embryonic development, cell proliferation or viability and the repair of γ-radiation induced damage.

People with mutations in Brca2 are predisposed to breast cancer (Wooster, *et al.*, 1994, Nature *265*:2088-2090: Smith *et al.*, Nature Genet. *2*:128-131: Easton, *et al.*, 1993, A. J. Hum. Genet. *52*:678-701). Neoplasia is associated with loss of heterozygosity of the non-mutated allele in tumors, suggesting Brca2 is a tumor suppressor. Brca2 is a 3,418 amino acid protein with no significant homology to any other genes (Wooster, *et al.*, 1995, Nature *378*:789-792; Tavtigan, *et al..*, 1996, Nature Genet. *13*:120-122). The mouse Brca2 protein is 3,328 amino acids and the overall identity is 58% between mouse and human Brca2 (Sharan, *et al.*, 1997, Genomics *40*:234-241).

3.0. SUMMARY OF THE INVENTION

Brca2 is a tumor suppressor and mediates Rad51 function. Consequently, it is possible that absence of Brca2 destabilizes or reduces Rad51 function which in turn is mutagenic. Some of these mutations could promote cancer.

Mice and cells have been generated with subtle mutations that inhibit the direct or indirect association of Brca2 with Mouse Rad51 (MmRad51). Data described here demonstrate that a subtle mutation which removes only a small portion of Brca2 that associates, either directly or indirectly, with MmRad51 exhibit a phenotype that suggests partial function. These *brca2*-mutant cells are viable yet hypersensitive to ionizing radiation suggesting they are deficient in the repair of double strand breaks in DNA. In addition, embryonic fibroblasts undergo premature replicative senescence, similar to cells deficient for the Ku autoantigen (United States Patent Application Serial No. 08/695,866, filed August 8, 1996), another protein involved in the repair of double strand breaks.

An object of the present invention is to provide animal cells which express an altered form of Brca2 that is impaired for its ability to associate with MmRad51, either directly or indirectly.

An additional object of the present invention is to provide mammalian, preferably mouse, embryos or mammals, preferably mice, which express an altered form of Brca2 that has an impaired ability to associate with MmRad51, either directly or indirectly.

These and other objects of the present invention which will be apparent from the detailed description of the invention are exemplified by a mouse cell containing two chromosomal alleles of the *Brca2* gene, wherein at least one of said alleles contains a mutation that produces Brca2 having an impaired ability to directly or indirectly associate with MmRad51.

Another embodiment of the present invention, is a mutant mouse embryo which produces Brca2 that has been engineered to have an impaired ability to directly or indirectly associate with MmRad51.

4.0. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Targeting strategy for *Brca2* locus. **A.** Deletion of exon 27 (coding nucleotides 9420-9984) with targeting vector pMB2TVhprt. This targeted allele is called *brca2*^{lex1}. An *HPRT* selection cassette was flanked upstream (5') by a 5.4 kb *Apa1/Sma1* genomic *Brca2* fragment and downstream (3') by a 1.9 kb *HindIII/SmaI* genomic fragment; thus, creating a 2.5 kb deletion that removes all of exon 27 of *Brca2*. Positive selection, *HPRT* minigene; negative selection, *thymidine kinase* (*tk*) cassette; plasmid backbone (pKS, Stratagene), wavy line. Southern analysis is an *BglII* digest **B.** Deletion of most of exon 26 and all of exon 27 (coding nucleotides 9265-9984) with targeting vector pMB2TVneo. This allele is called *brca2*^{lex2}. A *neomycin phosphotransferase* (*neo*) selection cassette was flanked upstream (5') by a 4.6 kb *Apa1/Cla1* genomic *Brca2* fragment and downstream (3') by a 1.9 kb *HindIII/SmaI* genomic fragment; thus, creating a 3.3 kb deletion that removes most of exon 26 and all of exon 27 of *Brca2*. Positive selection, *neo* cassette; negative selection, *thymidine kinase* (*tk*) cassette; plasmid backbone (pKS, Stratagene), wavy line. Southern analysis is an *BglII* digest.

Figure 2. Exposure of control and *brca2*^{lex1}/*brca2*^{lex2} cells to genotoxic agents. Survival fractions (100% X number of colonies after exposure to genotoxic agent / number of colonies not exposed to genotoxic agent) were measured after 10,000 cells were plated onto a 10 cm plate and colonies counted 10 days later. **A.** Dose response curve to γ-radiation. Controls are wild-type Hprt positive cells (AB1, one clone), wild-type Hprt negative cells (AB2.2, three clones), *brca2*^{lex1}/+ cells (eight clones) and *brca2*^{lex2}/+ (six clones). Each of these groups of control clones resulted in the same survival fraction and are averaged for this curve. Eight clones of *brca2*^{lex1}/*brca2*^{lex2} cells were averaged. **B.** Dose response curve to ultraviolet light. The average of three *brca2*^{lex1}/+ clones and two *brca2*^{lex2}/+ clones are

presented for controls. The average of five *brca2*^{lex1}/*brca2*^{lex2} clones are presented.

Figure 3. Growth characteristics of *brca2*^{lex1}/*brca2*^{lex2} embryonic fibroblasts. Mouse embryonic fibroblasts (MEF) were isolated from wild-type E15.5 day 129SvEv embryos and *brca2*^{lex1}/*brca2*^{lex2} MEF were isolated from E15.5 day chimeric embryos (129SvEv cells injected into Swiss Webster blastocyts). *brca2*^{lex1}/*brca2*^{lex2} MEF were isolated from embryos with black eyes by selection in 90 mM G418 for 10 days. *brca*^{2lex1}/*brca2*^{lex2} MEF were maintained with and without G418 selection for all experiments (presence or absence of G418 did not affect growth). All experiments begin with passage 1 cells. MEF were grown in M10 (10% fetal calf serum from HyClone, Dulbecco's Modified Eagle's Medium from GibcoBRL, 2 mM L-Glutamine, 49.5 U/ml Penicillin and 38.8 μg/ml Streptomycin). **A.** Growth curve. 8 X 10^4 MEF were plated onto eight 3.5 cm plates and individual wells of cells were trypsinized and counted over 11 days. **B.** Percentage of cells in S phase. MEF (4 X 10^5) were grown on a 6 cm plate for 2 days. MEF were continuously exposed to 10 μM 5-bromo-2'-deoxyuridine (BrdU) and harvested over a 48 hour time course. Cells were permeabilized and exposed to fluorescently - labeled anti-BrdU antibodies as well as propidium iodide to stain DNA. A fluorescence activated sorter (FACS) analysis was performed on the cells (2 X 10^5 cells) to determine the percentage of cells that had incorporated BrdU (indicating DNA synthesis and thus cell cycle progression) at each time point. **C.** Colony formation at low density plating. For each clone, 5000 MEF were plated onto a 10 cm plates (three plates for each clone) and grown for 14 days. The colonies were stained with crystal violet and the number of colonies were counted. Colonies are >3 cells. **D.** Colony size distribution (CSD). The percentage of colonies with >15 cells are compared to the total number of colonies with >3 cells. **E.** Measurement of life span. The life span was determined by measuring the number of passages the MEF could undergo before proliferation stopped. MEF were plated onto three 6 cm

plates (1 X 10^5 cells/ plate). MEF were trypsinized every 3.5 days and the total number of cells counted. MEF were then plated back onto three 6 cm plates and the procedure continued until there was not enough MEF to plate onto three plates. Then 1 X 10^5 MEF were plated onto only two plates and finally only one plate. MEF were considered to be completely senescent when fewer than 1 X 10^5 cells remained. One clone of wild-types cells spontaneously immortalized.

5.0. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to the production of Brca2-impaired cells, and Brca2-impaired non-human animals. The non-human transgenic animals contemplated by the present invention generally include any vertebrates, and preferably mammals, which encode a *Brca2* homolog. Such non-human transgenic animals may include, for example, transgenic pigs, transgenic rats, transgenic rabbits, transgenic cattle, transgenic goats, and other transgenic animal species, particularly mammalian species, known in the art. Additionally, bovine, ovine and porcine species, other members of the rodent family, e.g. rat, as well as rabbit and guinea pig and non-human primates, such as chimpanzee, may be used to practice the present invention. Particularly preferred animals are rats, rabbits, guinea pigs, and most preferably mice.

Given the apparent similarity between the yeast and murine DSB repair mechanisms, the murine *Brca2* sequence utilized herein can be used as a heterologous probe to identify and isolate the corresponding genes from any of a wide variety of animal species. Typically, hybridization conditions are adjusted in accordance with the relatedness of the probe and target sequences. For example, hybridization/washing conditions should be of a lower stringency when the cDNA library (or target sequence) is derived from an organism different from the type of organism from which the labeled sequence was derived. With respect to the cloning of a *Brca2* homolog, using murine *Brca2* probes,

for example, hybridization can, for example, be performed at 65°C overnight in Church's buffer (7% SDS, 250 mM $NaHPO_4$, 2μM EDTA, 1% BSA). Washes can be done with 2XSSC, 0.1% SDS at 65°C and then at 0.1XSSC, 0.1% SDS at 65°C.

Low stringency conditions are well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.

Alternatively, the labeled *Brca2* nucleotide probe may be used to screen a genomic library derived from the organism of interest, again, using appropriately stringent conditions.

Further, a *Brca2* gene homolog may be isolated from nucleic acid of the organism of interest by performing PCR using two oligonucleotides designed from the *Brca2* sequences utilized herein. The template for the reaction may be cDNA obtained by reverse transcription of mRNA prepared from, for example, human or non-human cell lines or tissue, such as choroid plexus, known or suspected to express a *Brca2* gene allele.

The PCR product may be subcloned and sequenced to ensure that the amplified sequences represent the sequences of an *Brca2* gene. The PCR fragment may then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment may be labeled and used to screen a cDNA library, such as a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to isolate genomic clones via the screening of a genomic library.

PCR technology may also be utilized to isolate full length cDNA sequences. For example, RNA may be isolated, following standard procedures, from an appropriate cellular or tissue source (i.e., one known, or suspected, to express the obR gene, such as, for example, choroid plexus or brain

tissue). A reverse transcription reaction may be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" with guanines using a standard terminal transferase reaction, the hybrid may be digested with RNAase H, and second strand synthesis may then be primed with a poly-C primer. Thus, cDNA sequences upstream of the amplified fragment may easily be isolated. For a review of cloning strategies which may be used, see e.g., Sambrook et al., 1989, supra

Although virtually any animal cells may be utilized to practice the present invention, preferred embodiments of the present invention include diploid mouse cells, mouse embryos and mice that contain two chromosomal alleles of the *Brca2* gene, wherein at least one of the *Brca2*, alleles contains a mutation such said cell produces some Brca2 protein that is impaired with its function to associate, either directly or indirectly, with MmRad51. Such Brca2-impaired cells and mice are deemed to be useful as, *inter alia*, disease models for the analysis and testing of therapeutic agents, and the effects of mutagenic stimuli such as radiation and chemical mutagens.

Replicative or cellular senescence is a process common to cells that leads to their terminal arrest and probably functions as a control against tumor formation and may reflect organismal aging (Campisi 1996, Cell *84*:497-500). Given that Brca2-impaired cells, and possibly animals, exhibit features of accelerated senescence, the presently described cells and animals are also deemed to be useful for the study of biological aging, and agents for retarding the same.

In particular, methods are contemplated for the screening for compounds, conditions, or compensatory mutations, that partially or fully rescue the proliferation and or senescence abnormalities associated with Brca2-impaired cells. Examples of such conditions include, but are

not limited to, the over expression of transfected genes of endogenous genes, or the mutagenesis of genes and the like. Examples of such compounds include peptides, peptides analogues, antisense or aptameric oligonucleotides, organic molecules, including prostaglandins, and the like.

As discussed above, one embodiment of the present invention includes a mouse cell containing two chromosomal alleles of the *Brca2* gene, wherein at least one of said alleles contains a mutation such that said cell produces Brca2 having an impaired ability to associate with MmRad51. Additional embodiments of the present invention include non-human animal embryos, and non-human transgenic animals incorporating the *Brca2*-impaired cells.

As used herein,"*brca2*-impaired" means that at least one of the two wild-type *Brca2* chromosomal alleles has been mutated to encode Brca2 having an impaired ability to directly or indirectly associate with MmRad51 or any other protein that associates with MmRad51. *Brca2*-impaired products can be easily measured using standard molecular biology techniques. For example, one can measure altered *Brca2* messenger RNA levels by using reverse transcriptase polymerase chain reaction (RT-PCR) (see Figure 1). Thus, the term *brca2*-impaired also includes homozygous, as well as a heterozygous genotypes, although a homozygous genotype is preferable.

The mutation in the *Brca2* gene is preferably a deletion mutation that removes part or all of the nucleotides that codes for the domain that mediates an association, either direct or indirect, with MmRad51, although substitution mutations, frame shift mutations, and/or insertion mutations are included within the scope of the present invention. Substitution mutations can be prepared by site directed mutagenesis, as described by Hasty *et al.*, 1991, Nature *350*:243-246, so as to introduce a stop codon or other mutation near the region that codes for the domain that associates with MmRad51, either directly or indirectly so as to give rise to a truncated Brca2 protein product having an

impaired ability to directly or indirectly associate with MmRad51. Similarly, insertion mutations can be introduced within the Brca2 gene taking advantage of the convenient restriction sites therein, such as any of the exonic restrictions sites or other sites which are easily identified by exonic sequencing of the Brca2 gene and restriction mapping (Figure 1), and the techniques described by Hasty et al., 1991; Joyner et al., 1989. Another method of introducing an insertion or other mutation consists of infecting with a retrovirus which integrates in the Brca2 locus, thereby creating a mutated brca2 allele as described by von Melchner et al., Genes and Dev 6:919-927. However, the mutants of the present invention preferably lack part of the DNA sequence coding for Brca2 so that a defective brca2 allele is more likely made such that the produced Brca2 protein is impaired in its ability to directly or indirectly associate with MmRad51.

The coding region of the Brca2 gene is approximately 9984 bp in size. For the purposes of this present invention, the nucleotides encoding the Brca2 gene shall be numbered according to the gene bank accession # U65594. Deletion mutants can be produced by eliminating a DNA fragment from a coding region of the Brca2 gene so that proper folding or substrate binding of the Brca2 protein with MmRad51 or another protein in this complex is impaired. The size of the deletion may vary. Alternatively, deleting a single base pair or two base pairs or any number of base pairs from the coding region would could result in impaired activity. In the latter instance, a truncated polypeptide may be produced because polypeptide synthesis is aborted due to a frame shift-induced stop codon. For a general review of mutagenesis and mutation see "An Introduction to Genetic Analysis", 4th edition, 1989 (D. Suzuki, A. Griffiths, J. Miller, and R. Lewontin, eds.), W. H. Freeman & Co., N. Y., New York.

Still, changing a single base pair in the coding region of the brca2 gene could also be a mutation which, if

resulting in an amino acid change, could alter the proper folding of the Brca2 protein and thereby create an Brca2 - impaired activity. A single amino acid change so generated could also alter the affinity of Brca2 for its substrate and thereby result in impaired association with MmRad51 or another protein in this complex. Another alternative would be to generate a deletion or other mutation in the non-coding region of the *Brca2* gene which affected the proper splicing of the *Brca2* messenger RNA. Such a mutation could effectively create a mutant *Brca2* transcript which was missing an entire exon or several exons as compared to the wild type *Brca2* message. Another alternative is to delete a non-coding regulatory region to decrease expression of the *Brca2* gene. Alternatively, promoter sequences could be deleted or altered that would diminish transcription of the *Brca2* gene and reduced transcription could result in an insufficiency of protein such that Brca2 association with MmRad51, either directly or indirectly, is impaired.

It is also possible to alter the expression of a given gene by altering codon usage in the gene. Alterations of this sort preserve the amino acid sequence of the product while increasing or decreasing the levels of expression.

Antisense RNA transgenes may also be employed to partially or totally knock-out expression of specific genes (Helene., C. and Toulme, J., 1990, Biochimica Bioshys. Acta *1049*:99; Pepin *et al.*, 1991 Nature *355*:725; Stout, J. and Caskey, T., 1990, Somat. Cell Mol. Genet. *16*:369; Munir *et al.*, 1990 Somat Cell Mol. Genet. *16*:383, each of which is herein incorporated herein by reference).

"Antisense polynucleotides" are polynucleotides that: (1) are complementary to all or part of a reference target sequence, such as the sequence of *Brca2* gene, and specifically hybridize to a complementary target sequence, such as a chromosomal gene locus mRNA. Such complementary antisense polynucleotides may include nucleotide substitutions, additions, deletions or transpositions, so long as specific hybridization to the relevant target

sequence is retained as a functional property of the polynucleotide. Complementary antisense polynucleotides include antisense which can hybridize specifically to individual mRNA species and hinder or prevent transcription or RNA processing of the mRNA species and/or translation of the encoded polypeptide (Ching *et al.*, 1989, Proc. Natl. Acad. Sci. USA *86*:10006-10010; Broder *et al.*, Ann. Int. Med. *113*:604-618; Loreau *et al.*, 1990, FEBS Letters *274*:53-56; Holcenberg *et al.*, W091/11535; W091/09865; W091/04753; W090/13641; and EP 386563, each of which is incorporated herein by reference). An antisense sequence is a polynucleotide sequence of at least about 15 contiguous nucleotides in length, typically at least 20 to 30 nucleotides in length, and preferably more than 30 nucleotides in length that is substantially complementary to nucleotides to a target gene sequence, or sequences in a cell. In some embodiments, antisense sequences may have substitutions, additions, or deletions as compared to the complementary target sequence but as long as specific hybridization is retained, the polynucleotide will generally function as an antisense inhibitor of gene expression.

For the purposes of he present invention, the antisense sequence is complementary to an endogenous *Brca2* target gene sequence. In some cases, sense sequences corresponding to the *brca2* target region may function to suppress expression, particularly by interfering with transcription. Alternatively, an antisense polynucleotide will generally suppress *Brca2* expression at a post transcriptional level.

Given that antisense polynucleotides inhibit the production of polypeptide(s) in cells, they may further alter a non-human transgenic animal's capacity to produce Brca2.

Antisense polynucleotides may be produced from a heterologous expression cassette inserted into transgenic pluripotent embryonic stem cells which may subsequently be used to generate the presently described Brca2-impaired animals.

The gene modified animal cells of the present inventions can be prepared by any of several techniques that are well established in the art. In particular, techniques conceptually similar to those taught in U. S. Patent No. 5,464,764 issued to Capecchi nd Thomas on November 7, 1995, herein incorporated by reference, may be used. In general, Brca2-impaired cells may be engineered using the following steps:

(1) Constructing a targeting vector comprising a cloning vector and a DNA fragment containing at least one positively selectable marker gene (positive selection marker), flanked by two non contiguous regions of the mouse *Brca2* gene or genomic locus which are in the same 5' to 3' orientation to one another referred to as the regions of homology;

(2) Included in the targeting vector a negatively selectable marker gene (negative selection marker) adjacent to one of the regions of homology. This negatively selectable marker may increase the likelihood of recovering the desired homologous recombination event deleting a portion of the *Brca2* gene but it is not required;

(3) Transfecting wild-type *Brca2* mouse cells with the targeting vector of step (2);

(4) Screening or selecting for said marker(s) in the resulting transfected mouse cells of step (3); and

(5) Screening for Brca2-impaired mouse cells from those cells in step (4) which are found to contain or express said positive selection marker(s) and not express said negative selection marker(s).

The precise *Brca2* gene or gene locus sequences which must be present in the targeting vector of step (1) will depend on the sequences chosen for the deletion, and (2) the restriction nucleases to be employed in the engineering of the deletion mutant.

The specific regions of homology required in step (1) depend on the specifics of the deletion in the targeting vector. In general, the size of the homology regions used in the targeting vector will be at least about 400 bp, though

longer or shorter regions could be used. In general it is preferable to use homology regions of approximately 1.5 kb or greater to insure a high degree of targeting efficiency. The targeting vector described in detail in Figure 1, the 5′ and 3′ homology regions on both sides of the deletion were greater than 1.5 kb.

The size of the deletion may also vary and depends on the regions of homology used in the targeting vector. That is, since non-contiguous regions of homology are used in the targeting vector, that region in the wild-type allele which is located between the regions of homology constitutes the region to be deleted upon homologous recombination with the targeting vector. The region to be deleted in the present invention is approximately 2.5 kb for *brca2*^{lex1} and 3.3 kb for *brca2*^{lex2}; however, the exact size is not critical and either more or less could be deleted from the locus and still result in *brca2*-deficiency. It is preferable that the deletion include at least one exon or a portion of an exon of the *Brca2* gene so as to result in mutant *brca2* messenger RNA.

The particular positive and negative selection markers employed in the present invention are not critical thereto. Examples of preferred positive and negative selection markers are listed in Table 1. The positive selectable marker should be located between the regions of homology and the negative marker, if one is used, should be outside the regions of homology, either 5′ or 3′ to those regions as shown in Figure 1a and 1b. The regions of homology should be in the same 5′ to 3′ orientation to one another while the orientation of the positive and negative selectable markers are not critical. It is not critical to include a negative selectable marker, though this may increase the efficiency of targeting.

The positive selectable marker should be engineered to be functional in the transformed cells in which the gene targeting is being performed. Positive and/or negative selection markers are functional in the transfected cells if the phenotype expressed by the DNA sequences encoding such selection markers is capable of conferring either a positive

or negative selection characteristic for the cell that is expressing the sequence. The means by which the positive selectable marker gene is made functional is not critical to the present invention. Positive selection is accomplished by exposing the cells to an appropriate agent which kills or otherwise selects against cell not containing an integrated positive selection marker. Examples of such agents are listed in Table 1. The positive selectable marker gene may have a promoter driving its expression or it may be driven by the juxtaposition of transcriptional elements at the target locus with the positive selectable marker. This latter method requires that those transcriptional elements are active in the transformed cells.

The mutation engineered in the targeting vector can contain DNA sequences between the regions of *Brca2* gene homology in addition to a positive selection marker, e.g., an oligonucleotide linker, in place of the deleted *Brca2* DNA. The oligonucleotide linker is generally 8-10 nucleotides in length, but can be longer, e.g. about 50 nucleotides, or shorter, e.g. 4, 5 or 7 nucleotides. The preferred length of the oligonucleotide linker is about 20 to 40 nucleotides in length. The DNA sequence of the oligonucleotide linker is not critical.

The method of inserting the oligonucleotide between the regions of homology in the targeting vector DNA will depend upon the type of oligonucleotide linker used. Palindromic double stranded linkers containing one or more restriction nuclease sites in the oligonucleotide sequence (New England Biolabs) may be inserted by well known procedures (Maniatis *et al.*, 1982, *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, N. Y.). pMB9, pBR325, pKH47 (Bethesda Research Laboratories), Oligonucleotide linkers may also be inserted into deletions in plasmid DNA by tailing ends with complementary homopolymers using terminal transferase (Maniatis *et al.*, *supra*). Alternatively, an oligonucleotide linker may be inserted into a deletion in a plasmid by bridging, *i.e.*, through annealing of

TABLE 1
Selectable Markers for Use in Gene Targeting

Gene	Type	Selective Agents	Preferred Concentration of Selective Agent	Organism
neo	+	G418	50 - 1000 μg/ml	Eukaryotes
hyg	+	Hygromycin	10 - 1000 μg/ml	Eukaryotes
hisD	+	Histidinol	5 - 500 μg/ml	Animals
gpt	+	Xanthine	50-500 μg/ml	Animals
hprt	+	Hypoxanthine	0.01 - 10 mM	All
HSV-tk	-	Gancyclovir	0.05 - 200 μM	Animals
	-	FIAU	0.02 - 100 μM	Animals
hprt	-	6-thioguanine	0.1 - 100 μg/ml	All
gpt	-	6-thioxanthine	0.1 - 100 μg/ml	Animals
Diptheria toxin	-	None	None	Animals
Ricin Toxin	-	None	None	Animals
cytosine deaminase	-	5-fluorocytosine	10 - 500 μg/ml	All

oligonucleotides containing ends complementary to a cleaved plasmid's 3'-recessed and 3'-protruding cohesive ends, followed by filling in of the gap complementary to the oligonucleotide sequence with DNA polymerase (*e.g.* Klenow fragment). After subsequent ligation with T4 DNA ligase, closed circular DNA molecules can be regenerated. If the targeting vector is designed such that the deleted region interrupts an exon, by the judicious choice of oligonucleotide linker length and sequence, frame shift mutations and/or stop codons may be produced in the mouse *Brca2* gene, augmenting the effect of deletions within the mouse *Brca2* gene.

Site-directed mutagenesis may be used to simultaneously construct a specific deletion and insert a linker sequence by using single stranded oligonucleotide to "loop-out" the desired region of the target gene (Krogstad and Champoux 1990, J. Virol. *64 (6)*:2796-2801, herein incorporated by reference).

The mutation engineered in the targeting vector can contain DNA sequences between the regions of *Brca2* gene homology in addition to the positive selection marker, for example, splice acceptor sequences. Such sequences have been shown to facilitate aberrant splicing to create mutant message.

The DNA used as regions of homology should be derived from genomic DNA from the *Brca2* gene locus from the mouse or sequences that flank the *Brca2* gene locus. The strain of mouse from which the DNA derives is not important but preferably it should be the same as the strain of mouse from which the cells derived in which the gene targeting will be performed. Using DNA for the homology regions which is isogenic to the cells the cells in which the gene targeting will be performed may enhance the efficiency with which gene targeting is accomplished. The regions of homology may be derived from genomic libraries of mouse DNA which may be cloned into a variety of library vectors such as lambda phage vectors, cosmid vectors, plasmid vectors, p1 phage vectors,

yeast artificial chromosome vectors, or other vectors. Regions of homology to be used in the targeting vector could also be derived directly from genomic DNA using the polymerase chain reaction (PCR). This method relies on having some knowledge of the sequence of the *Brca2* gene which is published (Sharan and Bradley 1997, Genomics *40*:234-241). Regions of homology so derived could be subcloned directly into the targeting vector.

The particular cloning vector employed in the present invention to construct the targeting vector comprising two regions of *Brca2* homology separated by a positive selectable marker gene and an optional flanking negative selectable marker is not critical as long as the cloning vector contains a gene coding for a selective trait, e.g. drug resistance. Examples of such cloning vectors include pBR322 and pBR322-based vectors (Sekiguchi, 1983 Gene *21*:267), pMB9, pBR325, pKH47 (Bethesda Research Laboratories), pBR328, pHC79, phage Charon 28 (Bethesda Research Laboratories, Boehringer Mannheim Biochemicals), pKB11, pKSV-10 (P-L Biochemicals), pMAR420 (Otsuka, 1981) and oligonucleotide (dg)-tailed pBR322 (Bethesda Research Laboratories), pBluescript or similar plasmids (Stratagene), puc19_or similar plasmids (New England Biolabs).

The targeting vector comprising two regions of *Brca2* homology separated by a positive selectable marker gene and an optional flanking negative selectable marker could be cloned into other cloning vectors such as lambda phage vectors, cosmid vectors, plasmid vectors, p1 phage vectors, yeast artificial chromosome vectors, or other vectors. Another option is to prepare the components of the targeting vector synthetically by PCR and simply ligating each component into its proper position by choosing restriction endonuclease sites for ligation which insured proper orientation of the homology regions relative to each other, and to insure that the positive selectable marker was located between the regions of homology.

Cloning vectors, other than the ones described in figure 1, containing unique cloning sites which are useful in the present invention can be determined upon evaluation of restriction nucleases. Other restriction nucleases which can be employed to produce fragments containing the mouse *Brca2* gene, and thus other cloning vectors which can be useful in the present invention, are readily apparent from the mouse *Brca2* gene restriction map. In fact, many combinations of restriction endonucleases could be used to generate an *Brca2* targeting vector to mutate the *Brca2* gene. These regions of homology could be cloned into any of a large number of commercially available plasmids such as the pBluescript series (Stratagene), the puc series (New England Biolabs), or the pGEM series (Promega).

The specific host employed for growing the targeting vectors of the present invention is not critical. Examples of such hosts include *E. coli* K12 RR1 (Bolivar *et al.*, 1977, Gene *2*:95); *E. coli* K12 HB101 (ATCC No. 33694); *E. coli* MM21 (ATCC No. 336780); and *E. coli* DH1 (ATCC No. 33849). The preferred host in the present invention is DH5alpha (Life Technologies). Similarly, alternative vector/cloning systems could be employed such as targeting vectors which grow in *E. coli* or *Saccharomyces cerevisiae*, or both, or plasmid vectors which grow in *B. subtilus* (Ure *et al.*, 1983, Methods of Enzymology, "Recombinant DNA", vol. *101*, Part C, Academic Press, N. Y.).

The specific mouse cell which is mutated in the present invention is not critical thereto, and is preferably a precursor pluripotent cell. The term precursor means that the pluripotent cell is a precursor of the desired transfected pluripotent cell which is prepared in accordance with the present invention. The pluripotent cell may be cultured *in vivo* to form a mutant mouse (Evans *et al.*, 1981, Nature *292*:154-156). Examples of mouse cells which can be employed in the present invention include embryonic stem (ES) cells (preferably primary isolates of ES cells), such as AB1 or AB2.1. Primary isolates of ES cells may be obtained

directly from embryos, such as described for the EK.CCE cell line or for ES cells in general. The particular embryonic stem cell employed in the present invention is not critical thereto. Examples of such embryonic stem cells are AB 2.1, an *hprt*$^-$ cell line, AB 1, an *hprt*$^+$ cell line. Other selectable markers such as those outlined in Table I could be used in other stem cell lines.

The ES cells are preferably cultured on stromal cells, e.g., STO cells and/or primary embryonic fibroblast cells as described by Robertson, 1987, *In* "Teratocarcinomas and embryonic stem cells: a practical approach", E. Robertson, ed (Oxford: IRL Press), pp. 71-112. The stromal (and/or fibroblast) cells serve to reduce the clonal outgrowth of abnormal ES cells.

In order to obtain the Brca2-impaired mice of the present invention, the mutant embryonic stems cells are injected into mouse blastocysts as described by Bradley, 1987, *In* "Teratocarcinomas and embryonic stem cells: a practical approach", E. Robertson, ed (Oxford: IRL Press), pp. 113-151.

The particular mouse blastocysts employed in the present invention is not critical thereto. Examples of such blastocysts include those derived from C57BL6 mice, C57BL6Albino, Swiss outbred, CFLP, MFI or others. Mice heterozygous for the *brca2* mutant allele generated from the injected blastocyst can be screened for mutations in the *Brca2* gene, e.g., by Southern blotting using DNA probes for said mutation (Figure 1), or by PCR.

The mutant mice of the present invention can be intercrossed to obtain embryos homozygous for the mutation in the *brca2* gene, and/or can be crossed with other mice strains to transfer the *brca2* mutation into these other strains.

The following examples serve to more fully describe the manner of making and using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the

true scope of this invention, but rather are presented for illustrative purposes.

6.0. EXAMPLES

Embryonic stem cells were manipulated essentially as described by published procedures (Teratocarcinomas and embryonic stem cells: a practical approach, E. J. Robertson, ed., IRL Press, Washington, D. C., 1987; Zjilstra *et al.*, 1989, Nature *342*:435-438; and Schartzberg *et al.*, 1989, Science *246*:799-803, each of which is herein incorporated by reference).

DNA cloning procedures were carried out essentially as described in J. Sambrook, *et al.* in Molecular Cloning: A Laboratory Manual, 2d ed., 1989, and periodic updates thereof, Cold Spring Harbor Laboratory press, Cold Spring Harbor, N. Y., which is incorporated herein by reference).

Oligonucleotides were synthesized on an Applied Bio Systems oligonucleotide synthesizer according to specifications provided by the manufacturer.

6.1. Cloning of the Mouse *Brca2* Gene

The mouse *Brca2* gene was cloned from a mouse 129SvEv - strain genomic library. More specifically, a fragment of the *Brca2* gene was obtained using oligonucleotides based on sequence and reverse transcriptase polymerase chain reaction on RNA from mouse cells. The fragment of the mouse gene so obtained was subcloned into a plasmid vector pBluescript SK+ (Stratagene). A radiolabeled probe was made using that subclone of the *Brca2* gene. The probe was used to screen a mouse 129SvEv-strain genomic lambda phage library to identify phage containing the homologous mouse gene. Three positive phage were isolated, grown, and restriction mapping performed on the DNA inserts by standard techniques.

6.2. Construction of Targeting Vectors

To generate Brca2-impaired mice, two targeting vectors were constructed. pMB2TVhprt was used to generate the *brca2*^{lex1} allele (Figure 1a): the vector contains 5.4 kb of DNA homologous 5' to exon 27 of the mouse *Brca2* gene, and 1.9 kb of DNA homologous 3' to exon 27 of the mouse *Brca2* gene. This vector also contains a marker for positive selection (the *Hypoxanthine phosphoribosyltransferase, HPRT,* minigene cassette), and a marker for negative selection (the *thymidine kinase, tk,* gene).

More specifically, based on the restriction map generated, a region of homology upstream and downstream to exon 27 of the mouse *Brca2* gene. The upstream homology region was isolated by an *Apa1* and *SacI* digest (Figure 1a) which released approximately a 5.4 kb DNA fragment. The downstream homology region was isolated by an *HindIII* and *SmaI* digest (Figure 1a) which released approximately a 1.9 kb DNA fragment.

To prepare pMB2TVhprt, a 2.5 kb genomic fragment from *SacI* to *HindIII* and containing coding nucleotides 9420-9984 were removed and replaced with the positive selectable marker. To prepare a positive-negative selection targeting vector, the negatively selectable *tk* gene was added exterior to the 3' homology region. The *KpnI* site which was unique and used to cut the vector prior to transfection (Figure 1a).

To generate the *brca2*^{lex2} allele: the vector (pMB2TVneo) contains 4.6 kb of DNA homologous 5' to exon 26 of the mouse *Brca2* gene, and 1.9 kb of DNA homologous 3' to exon 27 of the mouse *Brca2* gene. This vector also contains a marker for positive selection (the *neomycin phosphotransferase* cassette), and a marker for negative selection (the *tk* gene).

More specifically, based on the restriction map generated, regions of homology upstream of exon 26 (including only a small fraction of the 5' part of exon 26) of the mouse *Brca2* gene and downstream to exon 27 of the mouse *Brca2* gene were used. The upstream homology region was isolated by an *Apa1* and *Cla1* digest (Figure 1b) which released a DNA

fragment of approximately 4.6 kb. The downstream homology region was isolated by an *HindIII* and *Sma1* digest (Figure 1b) which released a DNA fragment of approximately 1.9 kb.

To prepare pMB2TVneo, a 3.3 kb genomic fragment from *ClaI* to *HindIII* and containing coding nucleotides 9265-9984 was removed and replaced with the positive selectable marker. To prepare a positive-negative selection targeting vector, the negatively selectable *tk* gene was added exterior to the 3' homology region. A unique *KpnI* site was used to cut the vector prior to transfection (Figure 1b).

6.3. Transfection of Mouse Embryonic Stem Cells

Homologous recombination of the targeting vector with the *Brca2* genomic locus was effected in mouse embryonic stem cells deficient for Hprt activity (See Figure 1). More specifically, 10 μg of the positive-negative targeting vector obtained in section 6.2 above was transfected into 1 x 10^7 129SvEv mouse strain embryonic stem cells deficient for Hprt activity and the resulting cells were grown in HAT (Hypoxanthine, Aminopterin, Thymidine) selection media to select for those cells which were transfected with the targeting construct to generate the *brca2*^{lex1} allele. Negative selection against the *tk* gene was also applied using the drug FIAU so as to enhance selection for those cells which had undergone a homologous recombination event at the *Brca2* locus. Surviving colonies were screened by mini-Southern, as described by Ramirez-Solis, 1992, Anal. Biochem. *201:331-336*, using a fragment of DNA from the *Brca2* locus which was 3' to the region of homology of the targeting vector as probes so as to detect the double reciprocal homologous recombination event between the targeting vector and the *Brca2* locus in the chromosome of the ES cell. Genomic DNA was digested with *BglII* and separated by electrophoresis. The desired recombination event was detected using the 3' probe which revealed a mutant allele of 2.9 kb for the pMB2TVhprt vector after gene targeting and a mutant allele of 9.4 kb for the pMB2TVneo vector after gene targeting as compared to the wild

type allele of 6.0 kb. Many positive ES cell clones were identified as correct replacement events, with an approximate 2.5 kb genomic deletion after gene targeting with the pMB2TVhprt vector and a 3.3 kb genomic deletion after gene targeting with pMB2TVneo.

Clones of targeted ES cells with the *Brca2*^{lex1} allele were subsequently targeted with the vector to generated the *Brca2*^{lex2} allele. After transfection with 10 μg of vector the cells were selected in G418 selection media to select for those cells which were transfected with the targeting construct to generate the *brca2*^{lex2} allele. Negative selection against the tk gene was also applied using the drug FIAU so as to enhance selection for those cells which had undergone a homologous recombination event at the *Brca2* locus. Surviving colonies were screened by mini-Southern, as described by Ramirez-Solis, 1992, Anal. Biochem. *201:331-336*, using a fragment of DNA from the *Brca2* locus which was 3' to the region of homology of the targeting vector as probes so as to detect the double reciprocal homologous recombination event between the targeting vector and the *Brca2* locus in the chromosome of the ES cell. ES cell genomic DNA for the minisouthern was digested with restriction enzyme *BglII* (Figure 1a,b). In addition, many of these targeted clones were mutated at both *Brca2* alleles to generate *brca2*^{lex1}/*brca2*^{lex2} compound heterozygotes.

6.4 Generation of Brca2 - Impaired Mice and Embryonic Fibroblasts

ES cell clones representing the following genotypes, *brca2*^{lex1}/+, *brca2*^{lex2}/+, *brca2*^{lex1}/*brca2*^{lex2}, as obtained in section 6.3 above were injected into C57BL6 Albino host blastocysts as has been described by Bradley, 1987, *In* "Teratocarcinomas and embryonic stem cells: a practical approach", E. Robertson, ed (Oxford: IRL Press), pp. 113-151. Injected blastocysts were implanted into pseudopregnant females and chimeric offspring were born as demonstrated by the mixture of agouti and albino coat colors (agouti

contribution from the ES cell line and albino from the wild-type host embryos). Chimeric male mice were mated to wild-type C57BL6 Albino females and agouti pups were born indicating successful germline transmission of the ES cell component of the chimeric mouse, resulting in C57BL6 Albino/129SvEv hybrids (referred to as C57BL6/129 hybrids). At three weeks of age, the offspring from the chimeric crosses were screened for the mutant *brca2* alleles as described below.

Genomic DNA was isolated from the resulting mice. Then 10 μg of the resulting genomic DNA was digested with *BglII*, and subjected to Southern blot analysis using the 3' probe as described above for the minisoutherns. ES cell clones transmitted the mutant allele through the germline for both *brca2*^{lex1} and *brca2*^{lex2} alleles. A male and female mouse were identified heterozygous for the mutant allele.

The male and female mice found to be heterozygous for the *brca2* mutations were intercrossed. The chimeric mice were also bred to 129SvEv strain mice, in order to place the mutant allele on the 129SvEv strain background.

6.5. The *brca2*^{lex1} mutation is most likely not null

The messenger RNA was analyzed to determine whether the *brca2*^{lex2} allele produced an altered transcript. RT-PCR (reverse transcriptase-polymerase chain reaction) was employed with one primer in exon 26 of mouse *Brca2* gene and another in exon 3 of the *HPRT* minigene. A fusion transcript was detected such that the sequences found in exon 27 of mouse *Brca2* were deleted and exon 26 sequences of mouse *brca2* were fused to exon 3 of the *HPRT* minigene. An additional amino acid is coded by the *HPRT* minigene sequences before a stop codon terminates translation (Figure 1c).

6.6. *brca2*^{lex1}/*brca2*^{lex2} Compound Heterozygous ES Cells are Hypersensitive to Ionizing Radiation but not UV light

The *brca2*^{lex1}/*brca2*^{lex2} compound heterozygous cells were tested for their ability to repair damage caused by two genotoxic agents, γ-radiation and UV light (Figure 2). For the γ-radiation analysis (Figure 2a): controls were wild-type Hprt positive cells (one clone), wild-type Hprt deficient cells (three clones), *brca2*^{lex1}/+ cells (eight clones), *brca2*^{lex2}/+ cells (six clones). No difference was found between these clones so their numbers were averaged. Eight clones of *brca2*^{lex1}/*brca2*^{lex2} cells were observed and averaged. At 250 RADS, 500 RADS and 750 RADS there were 2.5, 5, and 10 fold fewer *brca2*^{lex1}/*brca2*^{lex2} colonies to survive as compared to controls. For the UV light analysis (Figure 2b): controls were *brca2*^{lex1}/+ cells (three clones) and *brca2*^{lex2}/+ cells (two clones). No difference was found between these clones so their numbers were averaged. Five clones of *brca2*^{lex1}/*brca2*^{lex2} cells were observed and averaged. Both control and *brca2*^{lex1}/*brca2*^{lex2} cells exhibited the same degree of sensitivity to UV light. Therefore, the *brca2*^{lex1}/*brca2*^{lex2} genotype exhibits increased sensitivity to an agent that induces breaks in DNA (γ-radiation) but not to an agent that induces pyrimidine dimers (UV light).

6.7. *brca2*^{lex1}/*brca2*^{lex2} Compound Heterozygous Embryonic Fibroblast Cells Undergo Premature Replicative Senescence

Mouse embryonic fibroblasts (MEF) were analyzed for proliferation and life span (figure 3). Control MEF were derived from 129SvEv embryos. *brca2*^{lex1}/*brca2*^{lex2} MEF were derived from chimeric 15.5 day embryos (Swiss Webster recipient embryos injected with *brca2*^{lex1}/*brca2*^{lex2} 129SvEv cells). Chimerism was identified by embryos with black eyes since Swiss Webster is albino. *brca2*^{lex1}/*brca2*^{lex2} MEF were isolated from chimeric embryos by growing in G418 for ten days which selected for expression of the *neo* cassette. The proliferation characteristics was determined for control and

brca2^{lex1}/*brca2*^{lex2} MEF. To ensure the *brca2*^{lex1}/*brca2*^{lex2} MEF were not contaminated with cells derived from the Swiss embryo, they were grown with and without G418 (there was no contamination because no difference in proliferation was observed).

A growth curve was established for control and *brca2*^{lex1}/*brca2*^{lex2} MEF plated at high density (8 X 10^4 cells/ 3.5 cm plate). The control MEF grew slightly faster than *brca2*^{lex1}/*brca2*^{lex2} MEF at high density (Figure 3a) indicating that the *brca2*^{lex1}/*brca2*^{lex2} suffered from a growth disadvantage. MEF were labeled with BrdU and stained with propidium iodide to measure the number of cells that go into S phase over a period of 48 hours. About 10 - 20% fewer *brca2*^{lex1}/*brca2*^{lex2} MEF entered S phase than control MEF, indicating that a higher percentage of *brca2*^{lex1}/*brca2*^{lex2} MEF were senescent (Figure 3b).

Proliferative ability was established for control and *brca2*^{lex1}/*brca2*^{lex2} MEF plated at low density. The difference in proliferation was much more dramatic at low density (Figure 3c). The total number of colonies (includes all colonies with >3 cells) was counted and there were 10 fold fewer *brca2*^{lex1}/*brca2*^{lex2} colonies than control colonies when 5000 MEF were plated onto a 10 cm plate. A percentage of these colonies, ones with >15 cells, was determined for a colony size distribution (CSD). The CSD is an accurate indication of the cell's life span. Again there was a 10 fold decrease in the CSD for *brca2*^{lex1}/*brca2*^{lex2} MEF compared to control MEF (Figure 3d).

Life span of the control and *brca2*^{lex1}/*brca2*^{lex2} MEF was established (Figure 3e). The CSD indicates that the *brca2*^{lex1}/*brca2*^{lex2} MEF will undergo senescence faster than the control MEF. MEF were plated (1 X 10^5 cell/ 3.5 cm plate) onto three plates. They were passaged every 3.5 days and replated at the same concentration. As the number of cells decreased, then the same number of cells was plated onto fewer plates until there were no longer enough cells to plate onto a single plate. At this point the cells are considered

senescent. The *brca2*^{lex1}/*brca2*^{lex2} MEF were shown to become senescent at passage 7 - 8 while the control MEF could be passaged longer. In addition, one control MEF spontaneously immortalized. Thus the *brca2*^{lex1}/*brca2*^{lex2} MEF undergo premature replicative senescence.

6.8. Screen for rescue of proliferation/senescence defect in Brca2 - impaired cells

Embryonic fibroblasts impaired for Brca2 function will be used to screen for genetic mutations that rescue the proliferation/premature replicative senescence defect. The mutations may be made by a variety of techniques and the particular technique employed to make the mutations is not important. Examples of methods to make mutations is to expose the cells to DNA damaging agents, preferably agents that do not generated double-strand breaks because it is likely that double-strand breaks will be lethal to these cells. Another method is to infect with retrovirus. The integration of the retrovirus will introduce mutations.

Another approach to rescue the proliferation is to ectopically express transgenes in the fibroblasts impaired for Brca2 function. A variety of expression libraries may be used and the particular kind of library is unimportant.

Another approach to rescue the poor proliferation/ premature replicative senescence phenotype is to induce overexpression of endogenous genes in the fibroblasts impaired for Brca2 function. A variety of techniques may be used and the particular kind of technique is unimportant.

6.9. Mice and Cells Impaired for Brca2 Function as Animal Models for Cancer and to Test the Mutagenicity of Toxic Agents

Mice and cells that are impaired for Brca2 function may be used as a model system for oncogenesis and to test the mutagenicity of genotoxic agents. Brca2 impaired mice may be observe for onset and type of cancer. Brca2 impaired mice may be bred to mice with known predispositions to cancer, such as p53-mutant mice to observe a change in the onset of

cancer or the spectrum of cancer. Brca2-impair mice and cells may be exposed to a variety of toxic gent. o test for mutagenicity by onset and spectrum of tumor formation or by observing cell viability, proliferation and chromosomal damage.

All publications, patents, and patent applications mentioned in the above specification and herein incorporated by reference. Various modifications and variations of the described invention will be apparent to those skilled of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the above-described methods, techniques, and cells and animals are intended to be within the scope of the following claims.

WE CLAIM:

Claim 1. A diploid animal cell containing an engineered mutation in at least one allele of the *Brca2* gene.

Claim 2. The cell as of Claim 1, wherein the cell is homozygous for the mutant *brca2* allele.

Claim 3. The cell as of Claim 1, wherein said mutation is a deletion mutation.

Claim 4. A diploid animal cell containing an engineered mutation in a first allele of the *Brca2* gene and containing another mutation engineered in a second allele of the *Brca2* gene.

Claim 5. A non-human transgenic animal which comprises an engineered mutation which alters the expression or function of at least one allele of the *Brca2* gene.

Claim 6. A mutant embryo offspring of the non-human transgenic animal of Claim 5.

Claim 7. An animal of Claim 5 that is homozygous for said mutation in the *Brca2* gene.

Claim 9. An animal according to any one of Claim 5, wherein said mutation is a deletion mutation.

Claim 10. A method for screening for mutations that rescue the premature replicative senescent phenotype of the Brca2-impaired cells, comprising the steps of:

(A) growing genetically altered Brca2-impaired cells to observe cells that do not become senescent after passage 8;

(B) performing a 3T3 or 3T9 or population doubling analysis on cell that do not become senescent

after passage 8 to measure life span of the said cells;

(C) observing the morphology of cells that do not become senescent after passage 8 for characteristics found in senescent versus proliferative cells; and

(D) identifying the genomic alteration in the cells from step C.

Claim 11. A method according to Claim 10, said method further comprising the overexpression of transfected genes or endogenous genes.

Claim 12. A method according to Claim 10, said method further comprising the ectopic expression of transfected genes or endogenous genes.

Claim 13. A method according to Claim 10, said method further comprising incorporating the *brca2* mutation or *brca2* mutations, or any mutation that results in a deficiency or impairment of Brca2 function, into a genetic background that is deficient in negative regulation of the cell cycle, proficient in progression through the cell cycle, or that alters the life-span of a cell or organism.

Claim 14. A method according to Claim 10, said method further comprising exposing the Brca2-impaired cells to molecules, compounds or peptides.

Claim 15. A method for screening for genetic characteristics that increase the incidence of cancer in the Brca2-impaired animals, said characteristics drawn from the group consisting of:

(A) overexpression of a transgene or transgenes;

(B) overexpression of a endogenous gene or genes;

(C) ectopic expression of a transgene or transgenes;

(D) ectopic expression of an endogenous gene or genes;

(E) underexpression of a transgene or transgenes; and

(F) underexpression of an endogenous gene or genes.

Claim 16. A method for screening for genetic characteristics that decrease the incidence of cancer in the Brca2-impaired animals, said characteristics drawn from the group consisting of:

(A) overexpression of a transgene or transgenes;

(B) overexpression of a endogenous gene or genes;

(C) ectopic expression of a transgene or transgenes;

(D) ectopic expression of an endogenous gene or genes;

(E) underexpression of a transgene or transgenes; and

(F) underexpression of an endogenous gene or genes.

Claim 17. A method for screening for compounds or molecules that increase the incidence of cancer in the Brca2-impaired animals, comprising the step of exposing Brca2-impaired animals to said compounds and identifying a compound or molecule that increases the incidence of cancer in the Brca2-impaired animals.

Claim 18. A method for screening for compounds or molecules that decrease the incidence of cancer in the Brca2-impaired animals, comprising the step of exposing Brca2-impaired animals to said compounds and identifying a compound or molecule that decreases the incidence of cancer in the Brca2-impaired animals.

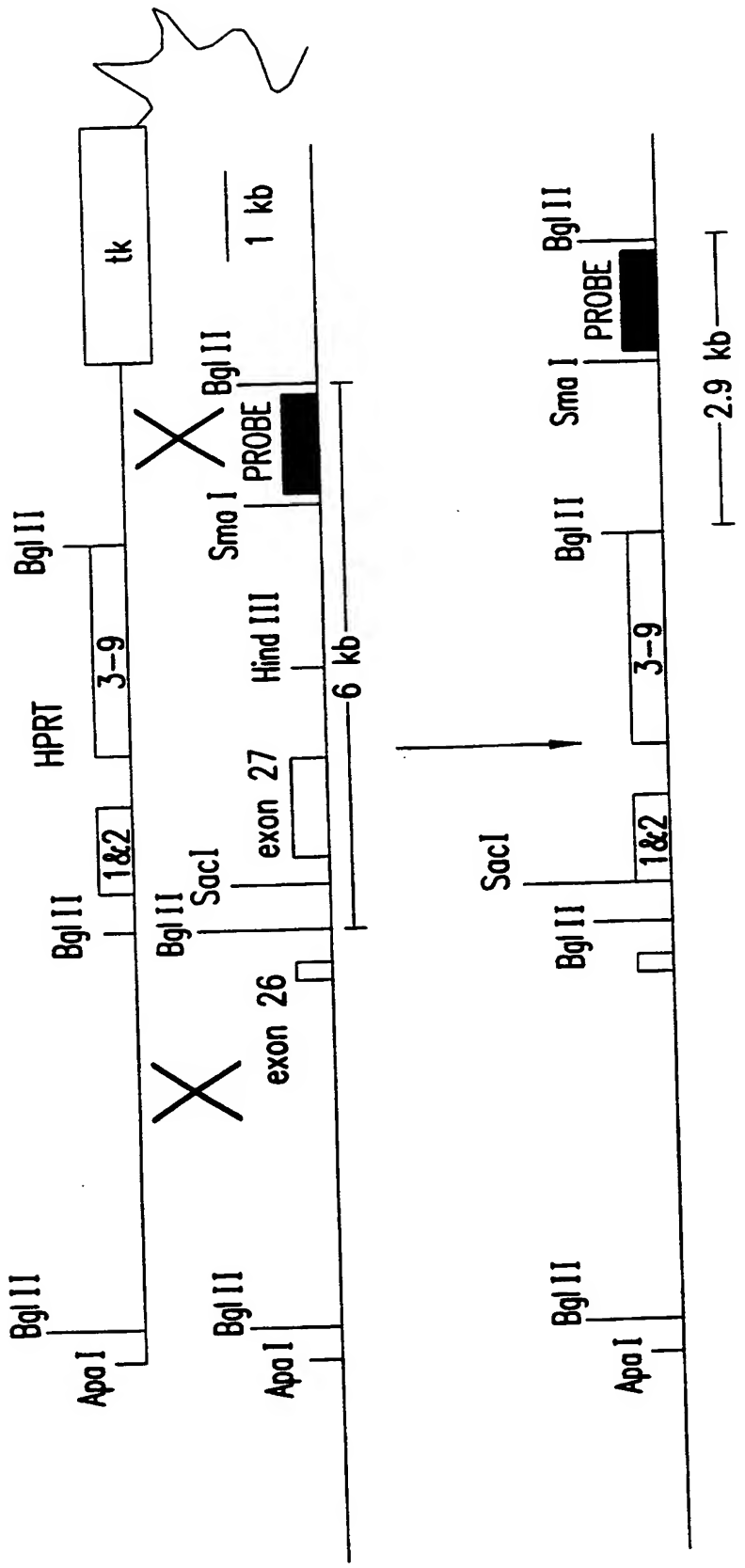
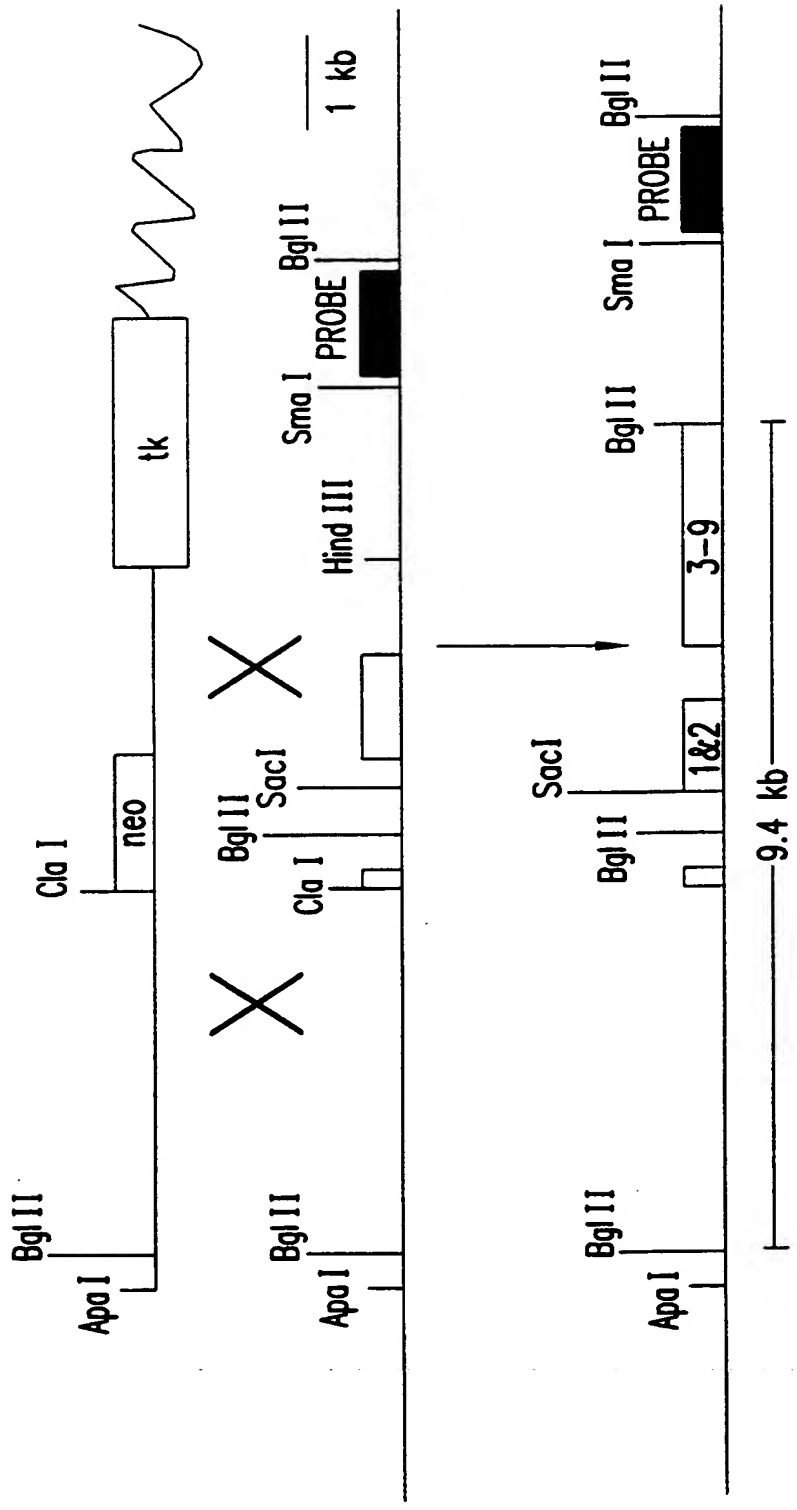


FIG. 1A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tk
neo
Cla I
Bgl II
Apa I
Bgl II
Sac I
Cla I
Hind III
Sma I
PROBE
Bgl II
Bgl II
Apa I
Sac I
Bgl II
1&2
3-9
Bgl II
Sma I
PROBE
Bgl II
Bgl II
Apa I
9.4 kb

FIG.1B

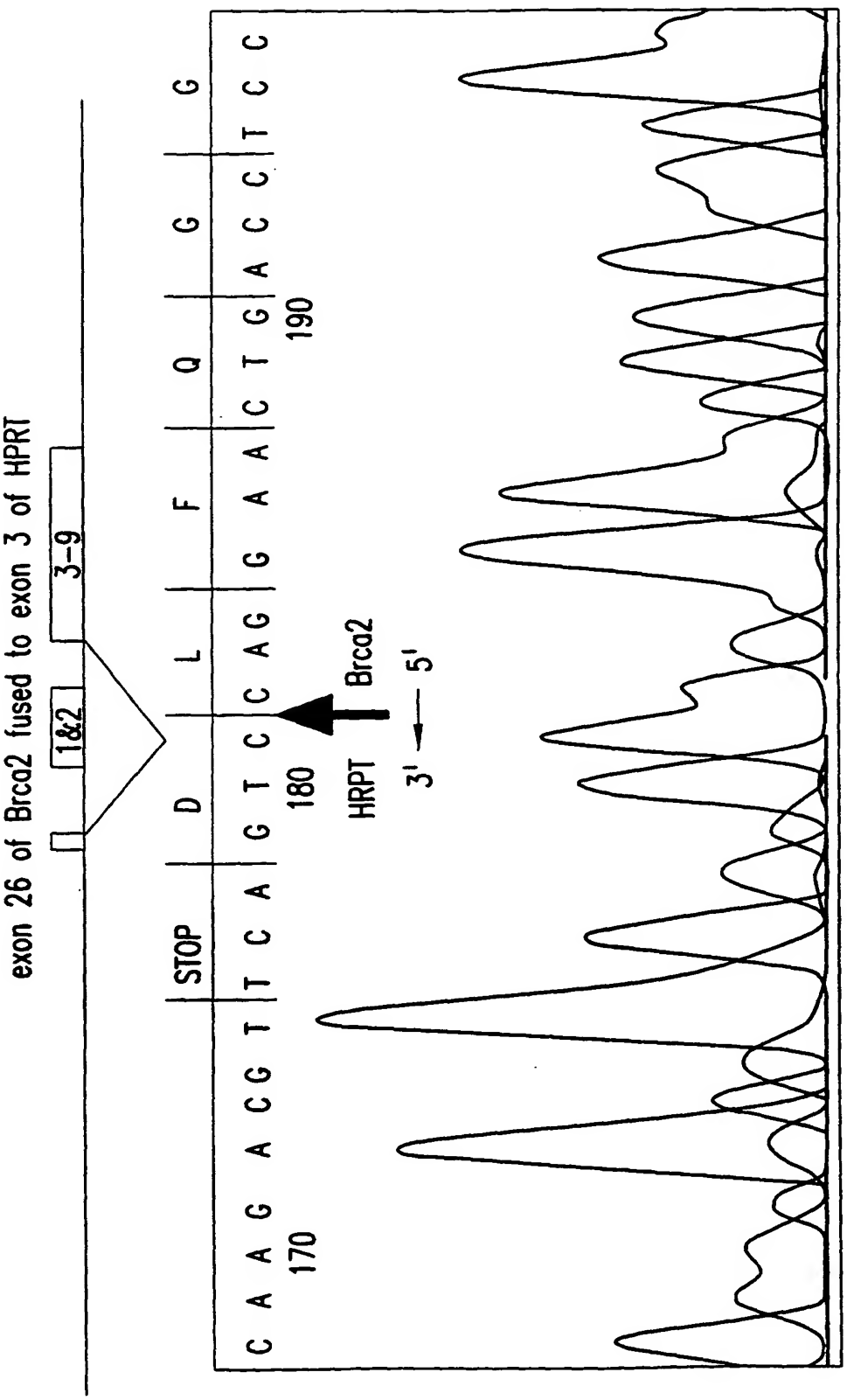

FIG.1C

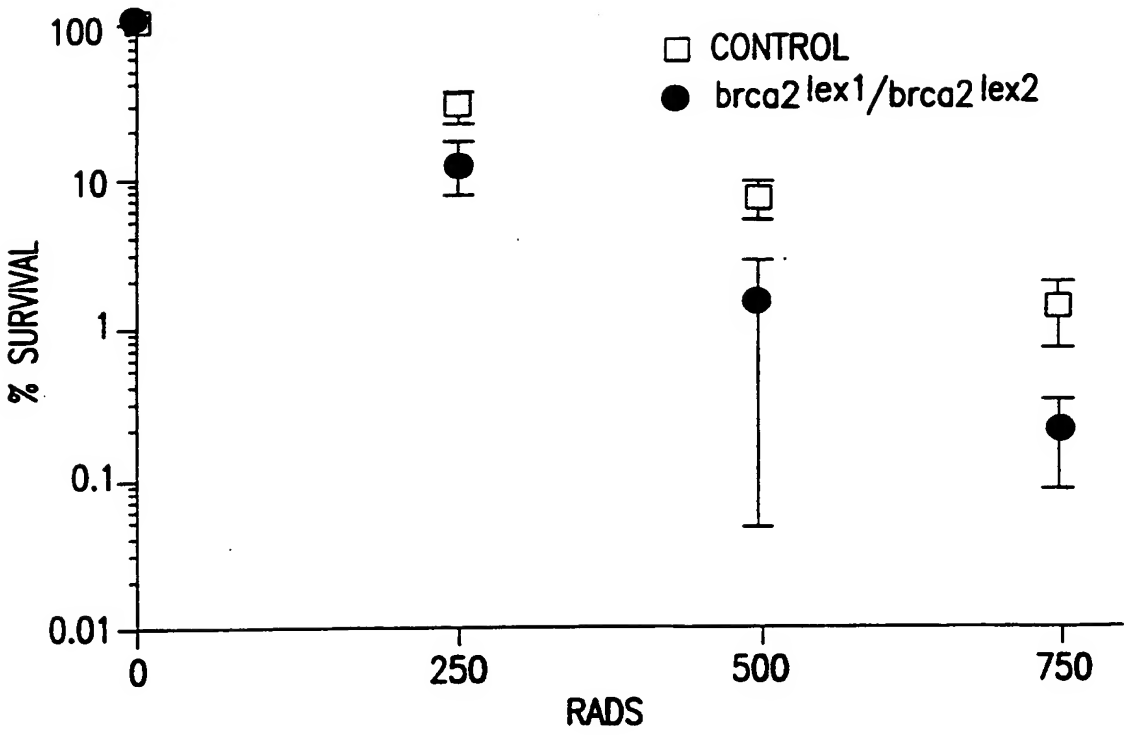

FIG.2A

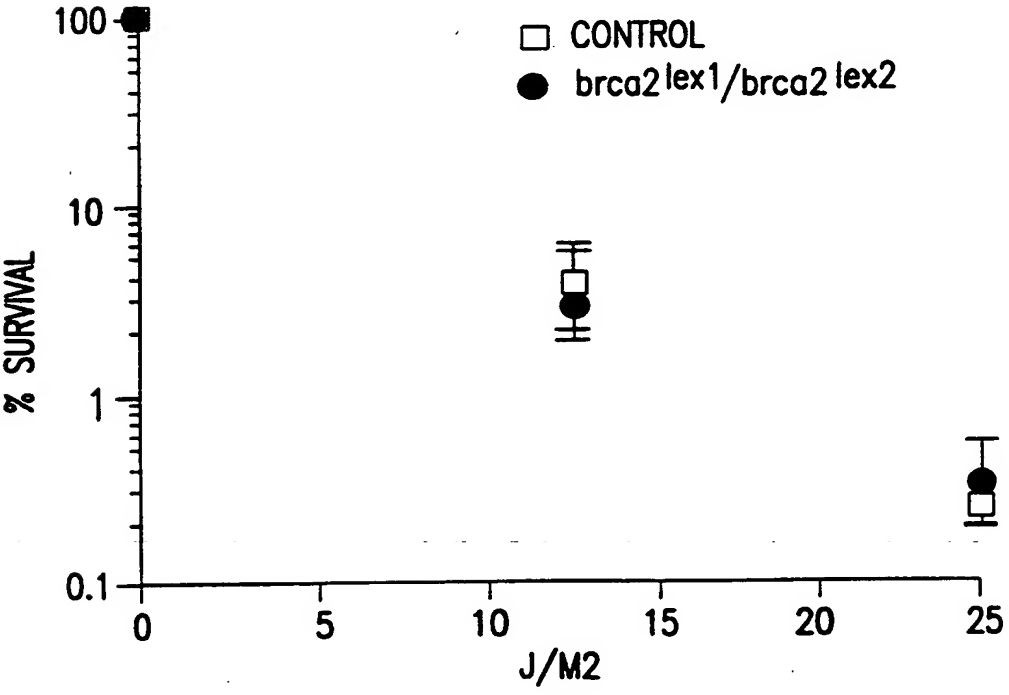

FIG.2B

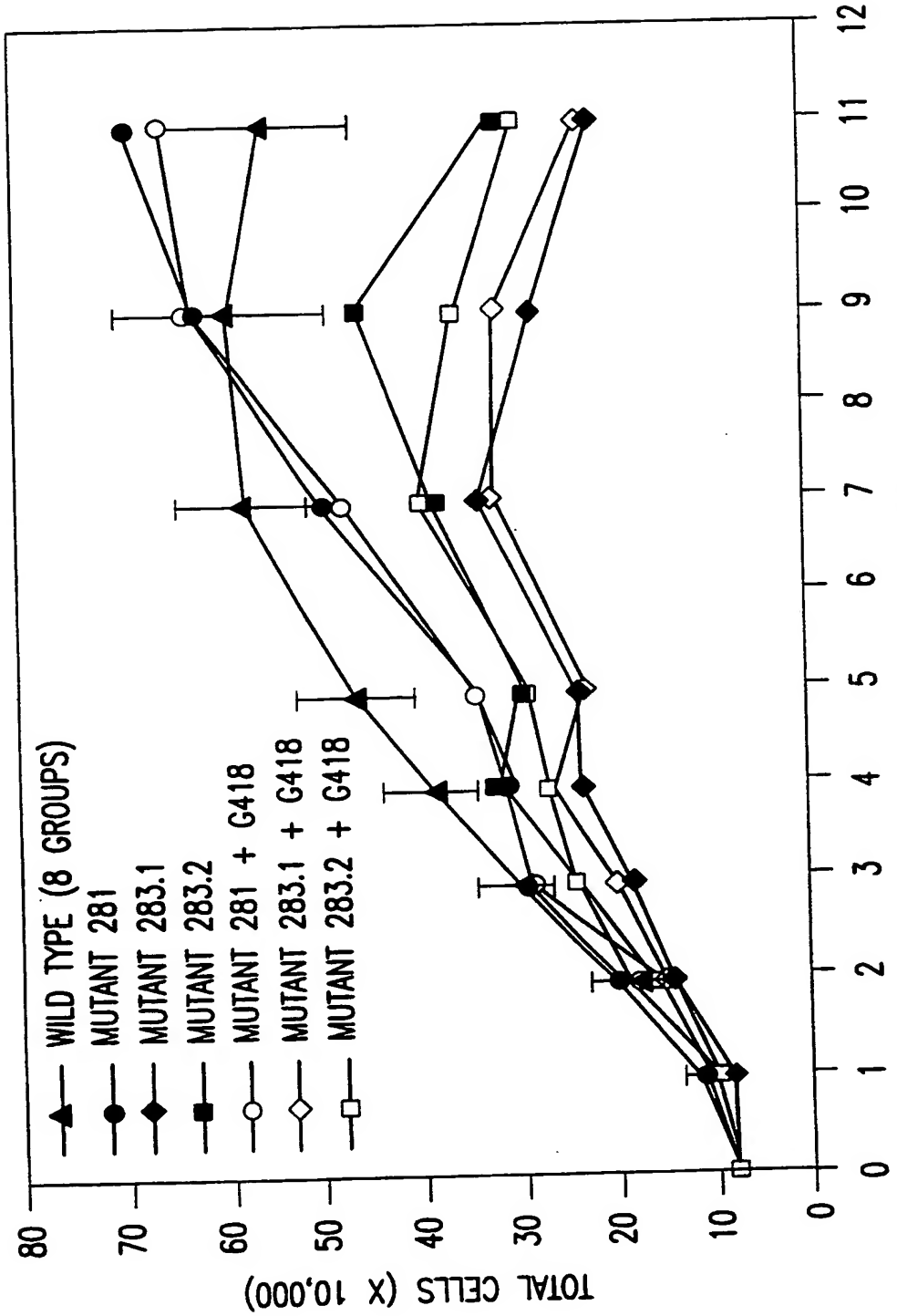

FIG.3A

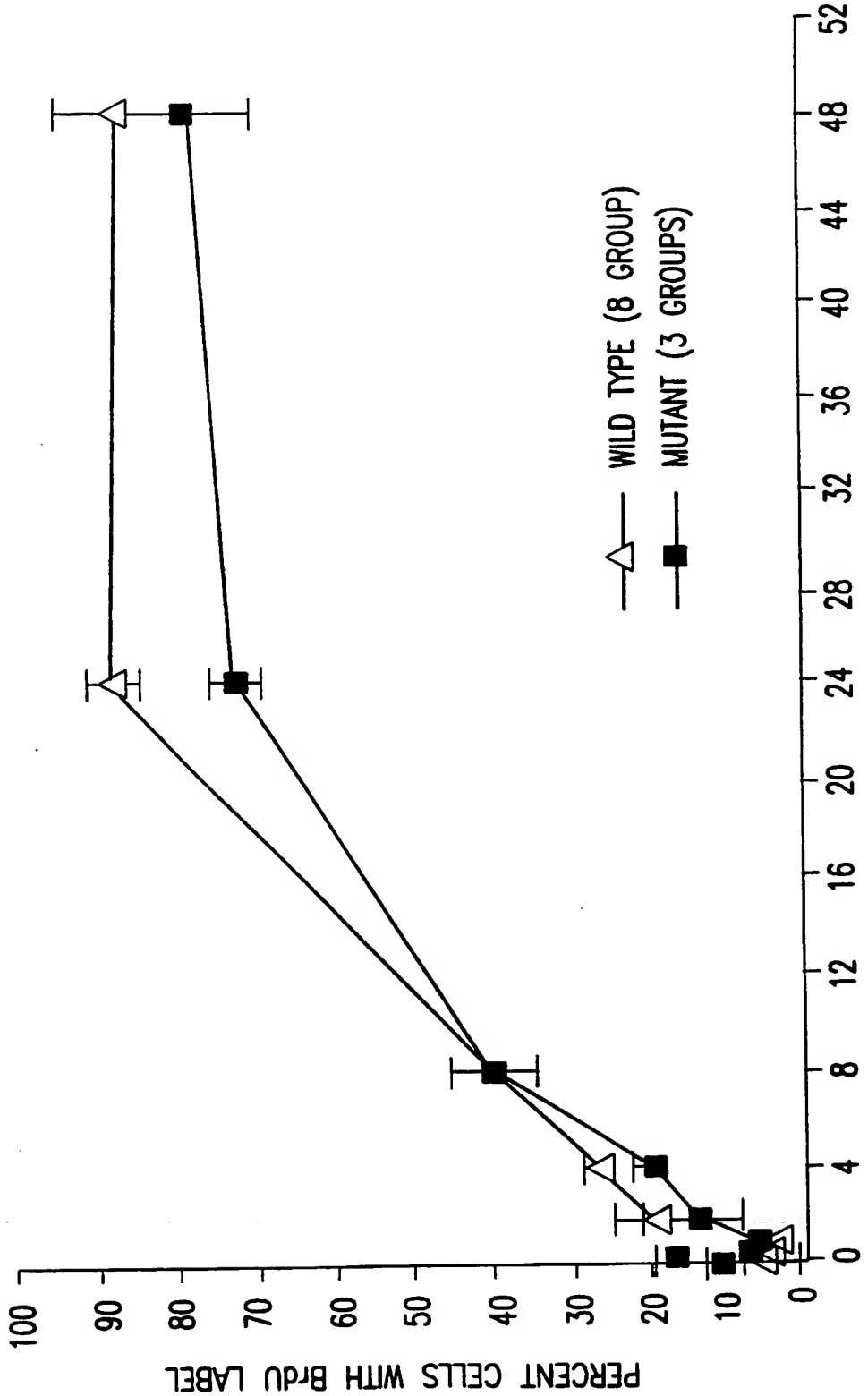

FIG.3B

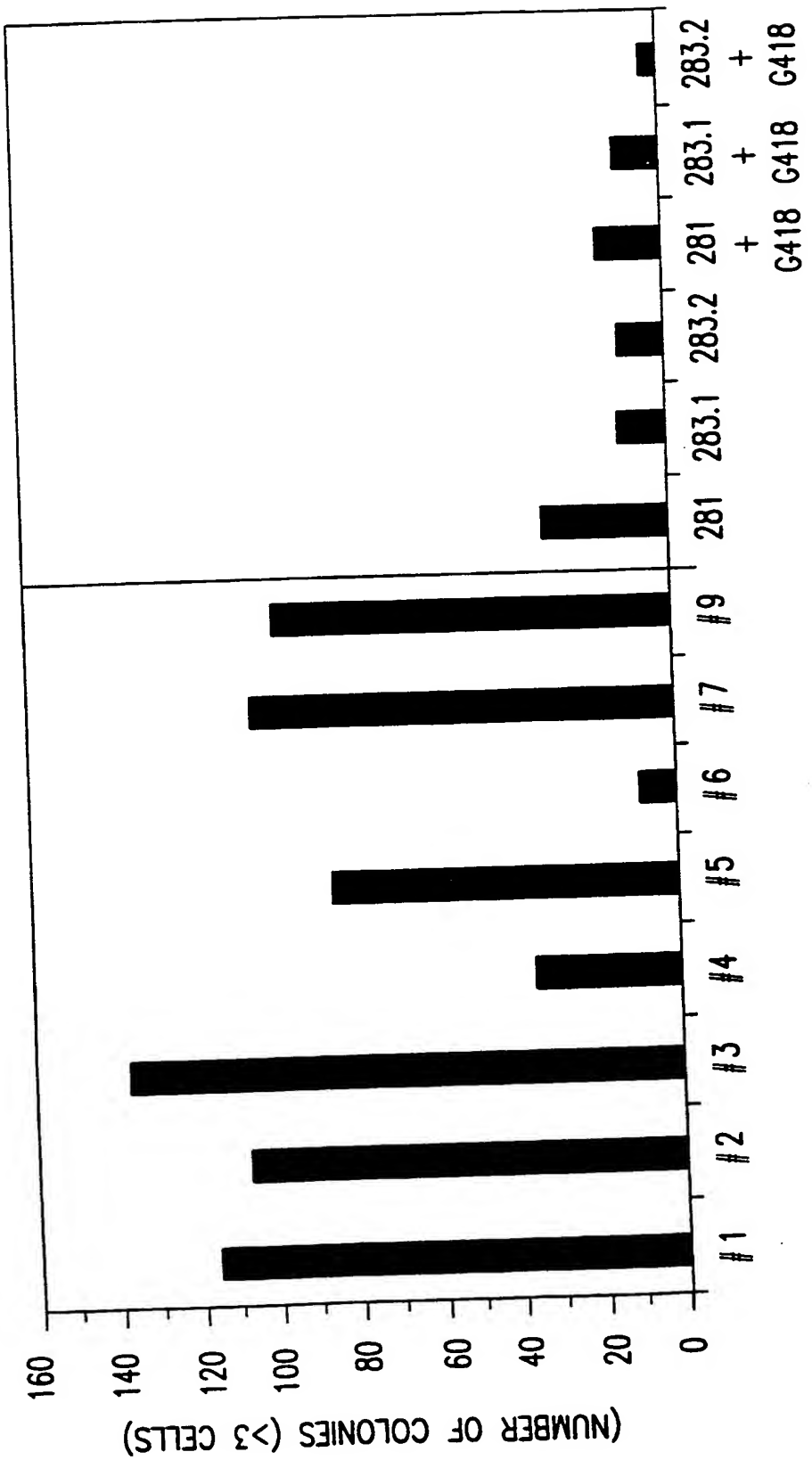

FIG.3C

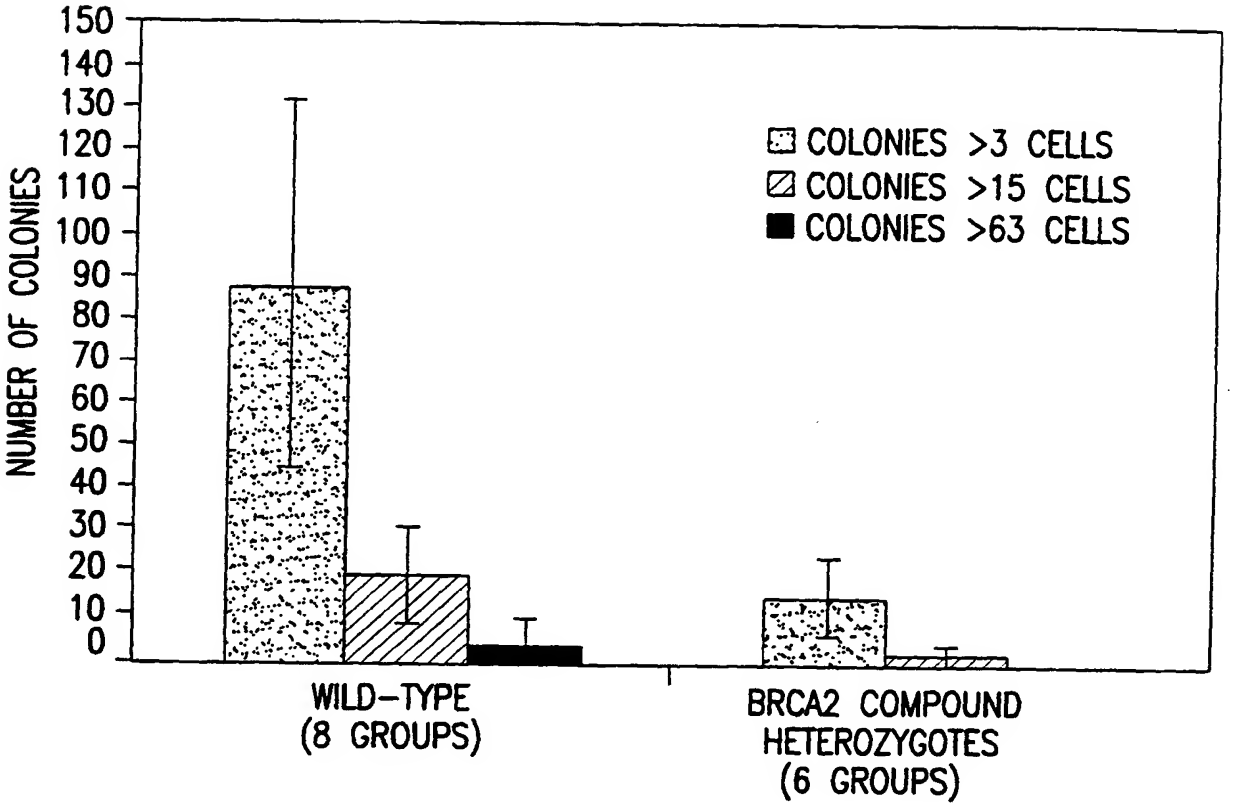
NUMBER OF COLONIES
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
COLONIES >3 CELLS
COLONIES >15 CELLS
COLONIES >63 CELLS
WILD-TYPE
(8 GROUPS)
BRCA2 COMPOUND
HETEROZYGOTES
(6 GROUPS)

FIG.3D

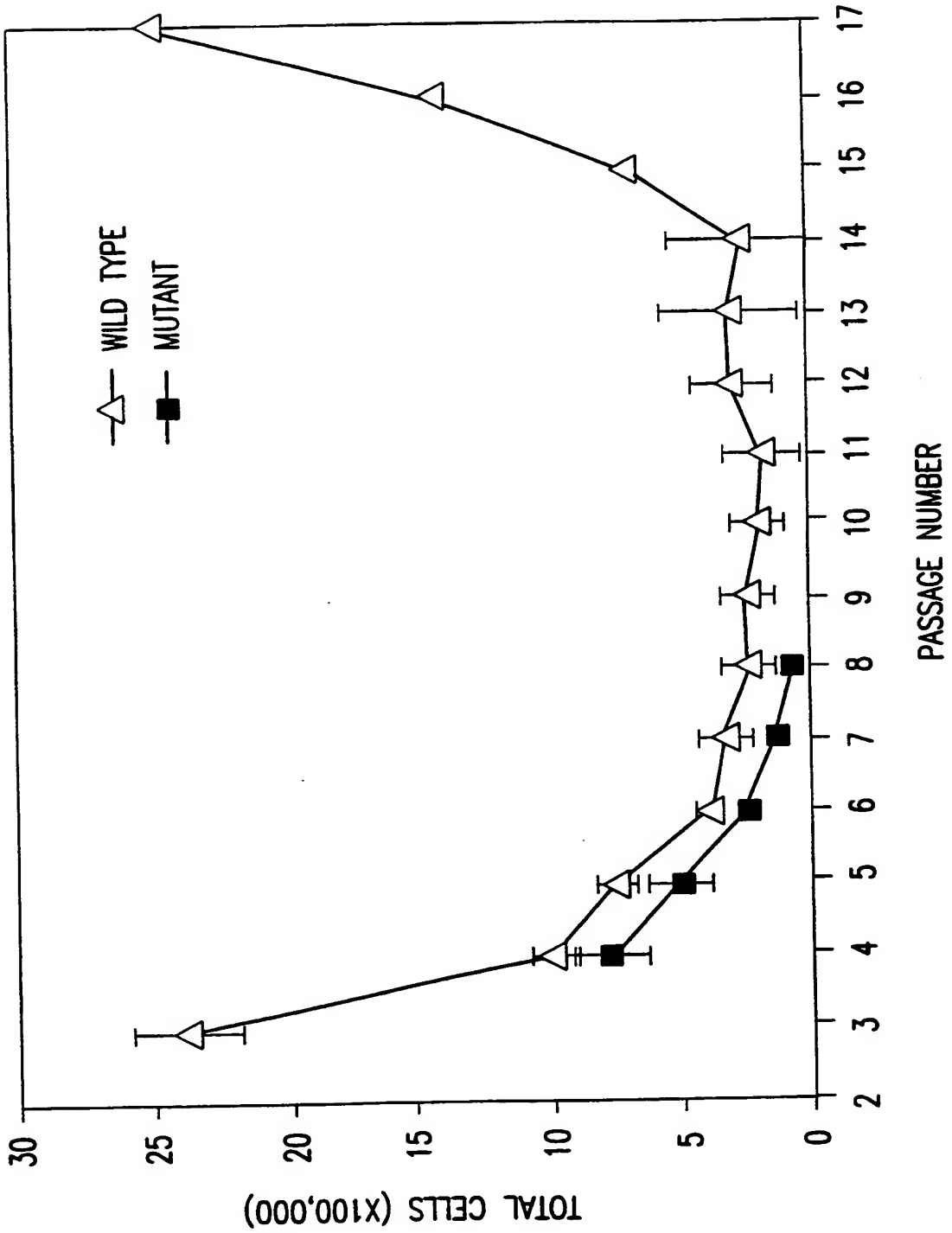

FIG.3E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17566

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00, 15/00, 15/09, 15/63
US CL :800/13, 21, 3; 435/6, 455, 463

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/13, 21, 3; 435/6, 455, 463

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, CAPLUS, BIOSIS, EMBASE, WPIDS, APS
search terms: brca2, mutation, transgenic, scrad51, replicative senescence

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MCALLISTER et al. Characterization of the Rat and Mouse Homologues of the BRCA2 Breast Cancer Susceptibility Gene. Cancer Research. 01 August 1997, Vol. 57, pages 3121-3125, see entire document.	1-18
Y	BRUGAROLAS et al. Double Indemnity: p53, BRCA and Cancer. Nature Medicine. July 1997, Vol. 3, No. 7, pages 721-722, see entire document.	1-18
Y	SHARAN et al. Embryonic Lethality and Radiation Hypersensitivity Mediated by Rad51 in Mice Lacking BRCA2. Nature. 24 April 1997, Vol. 386, pages 804-811, see entire document.	1-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 09 NOVEMBER 1998	Date of mailing of the international search report 22 DEC 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JILL D. MARTIN for Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17566

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LUDWIG et al. Targeted Mutations of Breast Cancer Susceptibility Gene Homologs in Mice: Lethal Phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 Nullizygous Embryos. Genes & Development. 15 May 1997, Vol. 11, No. 10, pages 1226-1241, see entire document.	1-18
Y	SHARAN et al. Murine Brca2: Sequence, Map Position, and Expression Pattern. Genomics. 01 March 1997, Vol. 40, No. 2, pages 234-241, see entire document.	1-18
Y	CONNOR et al. Cloning, Chromosomal Mapping and Expression Pattern of the Mouse Brca2 Gene. Human Molecular Genetics. February 1997, Vol. 6, No. 2, pages 291-300, see entire document.	1-18